APPLICANT:	INSTITUTE FOR RESEARCH INTO TISSUE REPAIR, REGENERATION AND RECONSTRUCTION
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AUSTRALIA

THE PATENTS ACT 1990

PROVISIONAL SPECIFICATION FOR THE INVENTION ENTITLED

"Method of Engraftment of Cultured Cells"

The invention is described in the following statement:

TITLE

METHOD OF ENGRAFTMENT OF CULTURED CELLS

FIELD OF THE INVENTION

The present invention relates to the use of cultured cells and a method of engraftment of the cells.

BACKGROUND OF THE INVENTION

The use of autologous skin grafts is a well established technique in the treatment of skin injuries, especially injury resulting from severe burns. With extensive burns the dermal and epidermal structures in particular areas may be so severely denuded that there is no potential for regrowth of the skin. Further, extensive damage to the skin practically limits the availability of healthy skin for regrafting.

In vitro cell culture techniques have been developed and refined to grow epidermal cells thus expanding the available epidermis for the purposes of skin grafting. The use of autologous cultured epidermal cells has become a recognised method for the coverage of wounds in the treatment of burns. For regeneration of skin the epidermal cells must be in a mitotically competent state (undifferentiated). Cells of the basal layer of the epidermis found adjacent to the dermis are undifferentiated and can undergo cell division and are therefore target cells.

The population of cultured and harvested epidermal cells form a new skin covering and initiate regrowth forming a natural skin substitute. One of the advantages being that the use of autologous cells eliminates major histoincompatability.

Methods of culturing epidermal cells are known in the art. US Patent Number 4,016,036 discloses a method for serially culturing keratinocytes using a feeder layer of irradiated 3T3 mouse fibroblasts. The keratinocytes are suspended on a layer of the lethally irradiated 3T3 cells, the keratinocytes divide and give rise to individual colonies of cells. The colonies of cells expand and displace the feeder cells to produce sheets of epithelium. US Patent Number 4,304,866 discloses a method of producing transplantable sheets of living keratinous tissue by culturing keratinocytes and utilizing an enzyme such as Dispase to remove the sheet from the surface of the culture vessel. Further, epidermal cells can be cultured using serum free systems.

Common culture techniques produce a sheet of epidermal cells. There are numerous disadvantages associated with the use of sheets of cultured epidermal cells. The sheets are thin and fragile and are therefore difficult to handle and manipulate. Cultured sheets are more difficult to cryopreserve. Further, the sheets take longer to grow, typically 12-14 days which may present undue delay for the management of a patient with severe burns. Furthermore, sheets do not conform well when used to cover a surface wound situated at particular sites such as an elbow or knee.

The present invention seeks to overcome at least some of the problems of the prior art by using cultured epidermal cells in suspension and providing a method for their engraftment during skin grafting procedures.

SUMMARY OF THE INVENTION

In accordance with one aspect of the present invention there is provided a method for

grafting cultured epidermal cells including the following steps:

a) obtaining viable epidermal cells by cultivation;

b) preparing a single cell suspension of a);

c) applying the single cell suspension as an aerosol to the wound site; and

d) dressing the wound site.

In accordance with a second aspect of the present invention there is provided a method for the treatment of burns in a human patient wherein a single cell suspension of cultured viable epidermal cells are sprayed on the wound site of a subject in need of such treatment.

In accordance with a third aspect of the present invention there is provided a delivery apparatus for medicinal use having a chamber , an atomiser member and a communication means therebetween, the chamber arranged to contain an epidermal cell suspension and the atomiser member arranged to reduce the suspension to a spray, whereby in use the cell suspension is dispensed from the chamber in aerosol form.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described, by way of example, with reference to the accompanying drawings in which;

Figure 1 is a schematic representation of a standardised method for the establishment of cultures of epidermal cells ; and

Figure 2 is a view of a delivery apparatus used to apply a suspension of cultured cells in accordance with the present invention.

DESCRIPTION OF THE INVENTION

The following description relates to the use of cultured autologous epidermal cells for engraftment of the cells during skin grafting procedures in the treatment of patients with extensive burns. It should be understood however that allograft material may be used. Further, graft material may be used in the treatment of other skin conditions.

With reference to Figure 1, a skin biopsy sample from an unburned area of a subject is obtained. Under aseptic conditions the biopsy is treated with a solution of antibiotics and then incubated in an enzyme solution which acts to facilitate separation of the epidermis from the dermis. The epidermis is then physically removed from the dermis and basal epidermal cells are harvested.

The harvested epidermal cells are suspended in a suitable culture medium and then cultured in vitro using known epidermal cell culture techniques. The epidermal cells are seeded into culture flasks at densities that will reach approximately 70-80 % confluence in approximately 6 - 8 days. The cell cultures are maintained under suitable temperature and atmospheric conditions with regular media change. When required for grafting the cell cultures are enzymatically treated to detach the epidermal cells from the surface of the culture flask and to disaggregate the cells to produce a substantially single cell suspension. It is to be understood that a small proportion of the cells may not fully disaggregate. Enzymatic action is arrested by the addition of Foetal Calf Serum (FCS) or other means.

The cell suspension is aliquoted into sterile containers for transport to theatre and maintained preferably at between 4-8 degrees. In theatre the cell suspension is transferred into a delivery apparatus 10, preferably the cell suspension is further diluted in cell culture medium. Referring to Figure 2 there is illustrated one embodiment of a delivery apparatus. The delivery apparatus 10 has a chamber 12 , an atomiser member 14 and a communication channel 16 therebetween, the chamber 12 arranged to contain a cell suspension and the atomiser member 14 is arranged to reduce the cell suspension to a spray, whereby in use the cell suspension is transported from the chamber 12 to the atomiser member 14 by passage through the communication channel 16 such that the cell suspension is dispensed in aerosol form.

The delivery apparatus 10 thus facilitates the application of the cell suspension in aerosol form.

In use, the cell suspension is sprayed over an area of the injured skin covering at least part of the said area. The method of application of the epidermal cells provides a substantially even layer of cultured epidermal cells in contact with the injured area. The area is then protected with a surgical dressing. Typically, the dressing includes applying a porous, pliable membrane over the injured skin. The pores of the membrane allowing drainage of exudate and blood. The membrane should not allow desiccation or maceration of the wound. A layer of gauze impregnated with an non-adherent material such as Vaseline ® is then applied. Further layers of surgical dressing may be applied in known manner providing a protective barrier against desiccation and infection.

The epidermal cells of the graft as applied in accordance with the present invention anchor at the wound site, the cells migrate and spread out over the wound and differentiate resulting in reepithelization of the wound.

The present invention provides an improved method for grafting of epidermal cells. Firstly, the method significantly reduces the amount of delay time between commencement of culture and grafting. The grafting can proceed at approximately day 6 to 8 rather than day 12 to 14, since there is no requirement to wait for confluent sheets of cells to form. The method minimises the handling of the fragile cultured cells. The method significantly reduces the time required to apply the graft to a subject in theatre which has obvious medical benefits to the patient as well as economic benefits such as savings in resources and efficient use of theatre time. Further the application in aerosol form allows even coverage and good conformity thus reducing the potential for scarring.

EXAMPLE

A preferred embodiment of the present invention is described as follows.

A $5cm^2$ skin biopsy sample is obtained from the patient, if the skin is thick the biopsy is cut into smaller pieces approximately $5mm^2$. The sample is incubated in an antibiotic solution (a mixture of approximately 50 IU/ml Penicillin, 50 µg/ml Streptomycin, 50µg/ml Gentamycin and 2.5 µg/ml Amphoterecin). The sample is then transferred and incubated in a pre warmed 0.5% trypsin solution, typically the skin is incubated for approximately 1 hour at 37°C in a dry incubator. The incubation time is dependent upon the thickness of the skin. The epidermis is then physically removed from the dermis.

Basal epidermal cells are then harvested from the exposed layers of the dermis and epidermis. The suspension of harvested cells is mixed and concentrated by centrifugation. The cell suspension is centrifuged at 1000 RPM for 5 minutes and the cells are resuspended in 10ml of cell culture medium. The cell culture medium consists of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12), 10% fetal bovine serum, 1.8 X 10^{-4}M adenine, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10}M cholera toxin and 10 ng/ml epidermal growth factor.

An aliquot of the cell suspension is counted to determine the concentration of the cell suspension. Epidermal cells are seeded at a concentration of approximately 3 X 10^6 per culture flask on a feeder layer of lethally irradiated 3T3 murine fibroblasts. The cell culture flasks are kept at 37°C in a humid atmosphere incubator with a CO_2 concentration of 5%. Media is changed regularly typically at day 2 and then on every second day thereafter. When the primary cultures reach approximately 70 to 80% confluence, usually after day 6 to day 8 the cells are harvested.

The cultures are trypsinized with 0.125% trypsin/0.05% EDTA solution at 37°C for between 15 to 30 minutes. The cells are washed with cell culture medium and the action of the trypsin is neutralised with the addition of fetal calf serum.

The cells are then washed twice with cell culture medium (no additives) to remove any foreign proteins.

A single cell suspension of the epidermal cells in cell culture medium is obtained and the cells are ready for use for engraftment.

The cell suspension is aliquoted into sterile containers in volumes of approximately 1ml and transported preferably at 4°C to theatre for use. In use the cell suspension is diluted in a ratio of 1:4 with 1ml cell suspension to 4ml of cell culture medium. In this embodiment the delivery apparatus comprises a syringe fitted with an atomiser nozzle at one end thereof. The diluted cell suspension is drawn into the syringe. The cell suspension is then sprayed over the injured site of the subject applying typically 1×10^5 cells per $75cm^2$ area.

The area is then protected with a surgical dressing. Firstly, a layer of SURFASOFT™, a monofilament polyamide mesh is placed over the sprayed injured site. JELONET ™, a Vaseline® impregnated gauze is then layered thereon. A further layer of BETADINE™ soaked gauze is then placed adjacent to the previous layer. Further layers of dry gauze and bandaging may then be used to dress the site.

Typically the injury site is clinically assessed after 5 days and if required the area may be resprayed with cultured epidermal cells in accordance with the present invention.

It is envisaged that the primary epidermal culture may be subcultured to obtain an increased number of cells for engrafting. Further it is envisaged that cultured epidermal cells may be cryopreserved using known methods and subsequently thawed prior to use. Furthermore it is envisaged that the cultured cells may be lyopholised. The lyophilised cells may be reconstituted for use. In this form the lyopholised cells may be packaged in kit form together with a delivery apparatus.

Modifications and variations such as would be apparent to a skilled addressee are deemed

within the scope of the present invention.

DATED THIS 3RD DAY OF OCTOBER 1996

INSTITUTE FOR RESEARCH INTO TISSUE REPAIR,
REGENERATION AND RECONSTRUCTION
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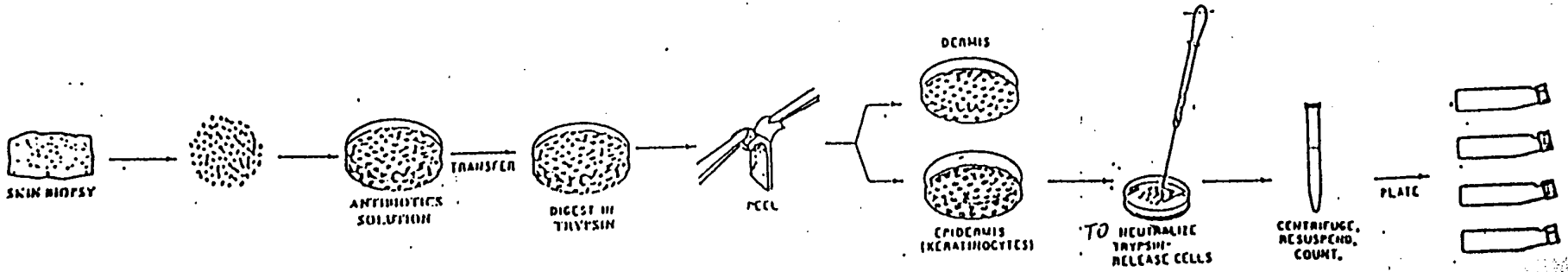
SKIN BIOPSY
ANTIBIOTICS SOLUTION
TRANSFER
DIGEST IN TRYPSIN
PEEL
DERMIS
EPIDERMIS (KERATINOCYTES)
TO NEUTRALIZE TRYPSIN- RELEASE CELLS
CENTRIFUGE, RESUSPEND, COUNT.
PLATE

FIGURE 1

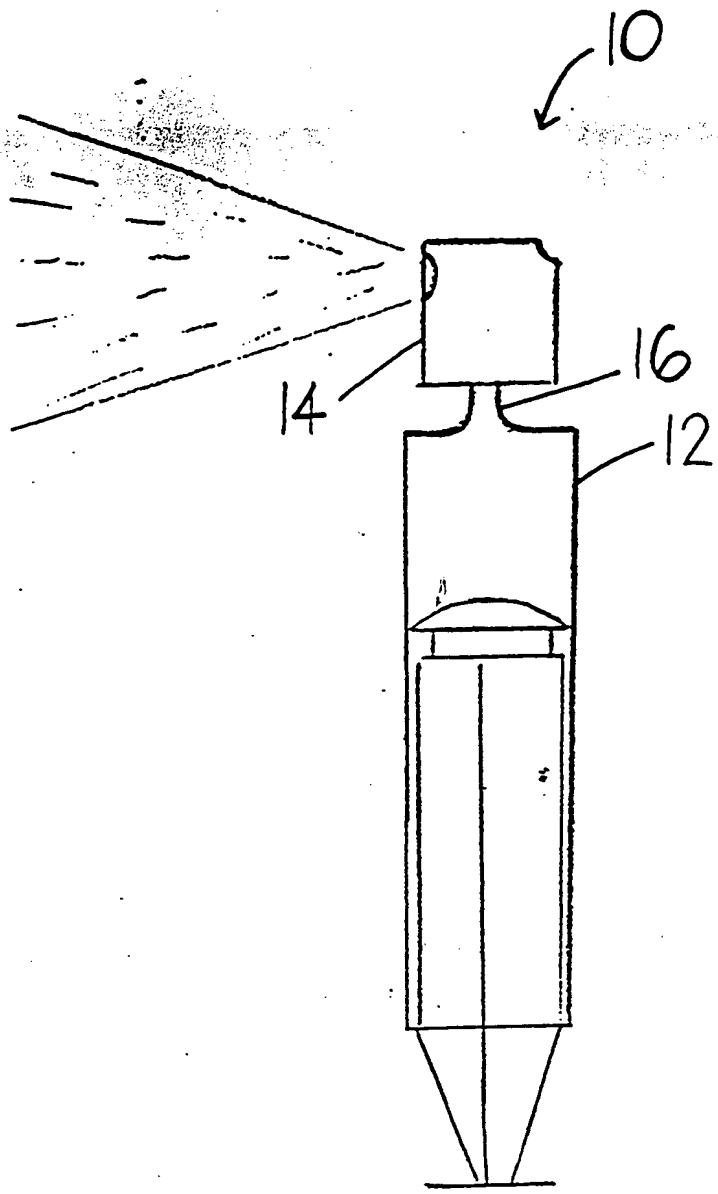

FIGURE 2